Contents lists available at ScienceDirect

Journal of Chromatography A

How changes in column geometry and packing ratio can increase sample load and throughput by a factor of fifty in Counter-Current Chromatography☆

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ARTICLE INFO

ABSTRACT

This paper builds on the fact that high aspect ratio rectilinear tubing columns of the same length and outside dimensions can double column efficiency. It demonstrates that further improvements in efficiency can be made by using rectilinear tubing columns with half the wall thickness thus replacing heavy PTFE with light solvent systems and producing lighter higher capacity columns. Increases in sample loading/throughput of up to 55x are demonstrated by comparing the separation of Honokiol and Magnolol using a Hexane: Ethyl Acetate: Methanol: Water (5:2:5:2) phase system with the new thin wall rectilinear column (56 mL, 30 mL/min, 2.1 g/h in 6.5 min.) with the original optimization performed using a conventional DE-Mini column (18 mL, 0.8 mm bore circular PTFE tubing, 2.5 mL/min, 0.038 g/h in 45 min.). Honokiol is currently going through first phase clinical trials as an anti-lung cancer therapy where preparative countercurrent chromatography was used for its manufacture. To be competitive in the future it is important for the technology to become more efficient. This is the first big step in that direction.

1. Introduction

The inspiration for this paper comes from the first phase clinical trial of Honokiol as an anti-lung cancer therapy [1]. Honokiol was manufactured using preparative countercurrent chromatography. For countercurrent chromatography to become competitive when scaling up for 2nd and 3rd phase clinical trials and final manufacture then the process must become more efficient. Honokiol and its scale up for manufacture was first reported by Chen in 2007 when a DE-Mini Instrument was used to maximize sample loading (0.038 g/hr) in preparation for quickly scaling up 3500x to the DE-Maxi-CCC Instrument (43 g in one 20-minute run) [2].

This paper utilizes the principle that increasing the aspect ratio of rectilinear tubing can double column efficiency compared to convention circular tubing [3]. It further increases column efficiency by using thin wall rectilinear tubing which increases the capacity of

the column by having a higher packing efficiency and has the additional advantage of producing a lighter column and therefore less wear and tear.

The objective of this paper is to see how far the new more efficient column geometry combined with better packing efficiency with thinner wall tubing can increase sample loading and throughput from what was possible with the standard 18 ml, 0.8 mm bore analytical instrument in 2007.

Berthod [4] has shown that increasing the bore of circular tubing can greatly increase stationary phase volume retention and allow faster separations with similar resolution despite the number of theoretical plates reducing dramatically. It will be interesting here to see if similar behaviour is observed with the more efficient rectilinear tubing.

The hexane ethyl acetate-methanol-water (5:2:5:2) phase system and the bobbin spool geometry were the same as used in [2].

2. Materials and methods

2.1. Experimental columns

Two experimental rectilinear columns were constructed, both wound on identical bobbins with the same internal and external

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dimensions. The first column (RH-ThickWall) has a rectangular section with its wide section horizontal relative to the radial force field which is perpendicular to it (h = 0.8 mm; w = 2.5 mm; AR = 3.125, Vc = 24.3 mL); the second column also has a rectangular section (RH-ThinWall) (h = 1.2 mm; w = 3.3 mm; AR = 2.75; Vc = 56 mL).

The outside cross-sectional dimensions of both rectangular sections are the same (h = 2.4 mm, w = 4.1 mm) the difference is in the wall thickness which is 0.8 mm for the thick wall tubing and 0.4 mm for the thin wall tubing. The cross-sectional area of the thick wall tubing is $2.0 \, mm^2$ equivalent to a bore in circular tubing of 1.6 mm. The cross-sectional area of the thin-wall tubing is $3.96 \, mm^2$ equivalent to a bore of 3.2 mm.

A bobbin wound with circular cross-sectional area tubing of 1.6 mm internal diameter (area = $2.0 \, mm^2$; Vc = 23.9 mL) is used for reference. The original commercial bobbin with circular cross-sectional area tubing of 0.8 mm internal diameter (area = $0.5 \, mm^2$; Vc = 18 mL) is also used for reference.

Rectilinear cross-sectional tubing was manufactured with the above dimensions by Adtech Polymer Engineering Ltd (Stroud, Glos.) in the UK and by Hongfa (Chengdu, Sichuan Province) in China. Bobbins were constructed using 3D printing technology (Viper Si2 stereolithography printer with Accura Xtreme photopolymer resin, South Carolina, USA) in the Advanced Bioprocessing Centre at Brunel University London.

2.2. Apparatus

The columns were mounted on a Mini-DE CCC centrifuge (Dynamic Extractions Ltd, Tredegar, Wales, UK), with a rotational planetary radius of 50 mm and a β value ranging from 0.54 to 0.76. A Knauer K-1800 HPLC pump (Berlin, Germany) was used to pump solvent into columns. A Knauer K-2501 spectrophotometer with a preparative flow cell was operated at 254 nm and 280 nm to monitor RP and NP elution respectively. For flows above 20 mL/min an Agilent 1200 Prep Pump was used.

2.3. Reagents

The solvent system used for the two-phase flow experiments was a HEMWat system 21 (Hexane/Ethyl Acetate/Methanol/Water – 5:2:5:2) [5]. The flow direction was Normal Phase (NP) (P > C). All solvents were of analytical grade from Fisher Chemicals (Loughborough, UK). HPLC water was purified by a Purite Select Fusion pure water system (Thame, UK).

2.4. Sample solution

The sample is a crude extract of *Magnolia officinalis* Rehd. Et Wils. The bark of magnolia was extracted with 95% ethanol. After recycling of the ethanol, the residue was re-dissolved in NaOH solution, after filtration, the solution was precipitated with HCl solution. The suspension was then filtered again and the residue was collected and washed by water before being dried in a vacuum at 40 °C.

Four grams of the above was made up to 50 mL with the upper mobile phase to give 80 mg/mL. This solution was used in 2.5%, 5%, 7.5% and 10% of column volume (CV) sample loops.

2.5. CCC separation procedure

The column was initially filled with the lower stationary phase, then the rotor speed was set at 2100 rpm (243 g), and the mobile phase pumped into the column to establish hydrodynamic equilibrium at the required flow in normal phase (NP – P > C) mode. Then the sample solution was injected and elution started, which was monitored with a UV detector at 254 nm. The volume of stationary phase eluted (Ve) was collected so that the volume retention of stationary phase (Sf) could be calculated in the usual way. As the sample loading could get quite high any stripping of stationary phase was noted and at the end of the run the contents of the column collected so that a check could be made on the final stationary phase volume.

2.6. Measurement of efficiency

The resolution (Rs) between honokiol and magnolol was used to assess separation efficiency.

2.7. Measurement of sample loading or throughput

Throughput was calculated as the weight of sample loaded divided by the separation time. So that comparisons could be made as flow and geometry varied, all throughput results were normalized to a resolution of 1.5 (ie complete baseline separation of Honokiol and Magnolol) by hypothetically changing the length of the column using the Rs~$\sqrt{L}$ relationship [6]

2.8. Calculation of g-Field

The values of g-field given in this paper are based on the g-field measured at the centre of the planetary rotor ($R\omega^2$) where R is the rotational radius of the planetary axis and ω is the angular rotation of the main centrifugal rotor. The acceleration field measured at the centre and periphery of where the tubing is wound on the bobbin will be much larger as described by van den Heuvel and Konig [7]

2.9. Repetitive experiments

Peak height variations were within 6–12%, whereas peak elution time/position was within ±2%.

3. Results

3.1. The variation of stationary phase retention for thick and thin walled columns

The variation of stationary phase volume retention (Sf) with the square root of mobile phase flow for normal phase (NP) is shown in Fig. 1 for both the thick wall (Vc = 24.3 mL) and thin-wall (Vc = 56 mL) columns. The effect of having a 10% column volume (10%CV) injection of sample can be seen to have little to no effect on stationary phase retention. Berthod [4] found for circular tubing when changing from 0.8 mm bore (0.5 mm²) tubing to 1.6 mm bore (2 mm²) tubing wound on the same bobbin spool that there was a huge increase in stationary phase volume retention (Sf). The same was found here when changing from the thick wall tubing (2 mm²) to the thin wall tubing (3.96 mm²).

3.2. The effect of increasing sample volume on baseline separation for the thick wall column

The effect of increasing sample volume from 2.5% of column volume to 10% of column volume for rectangular thick wall horizontal tubing is shown in Fig. 2. It can be seen that baseline resolution is preserved for 2.5%CV, 5%CV and 7.5%CV but at 10%CV this starts to be lost. Based on these results it was decided to use 10%CV for the
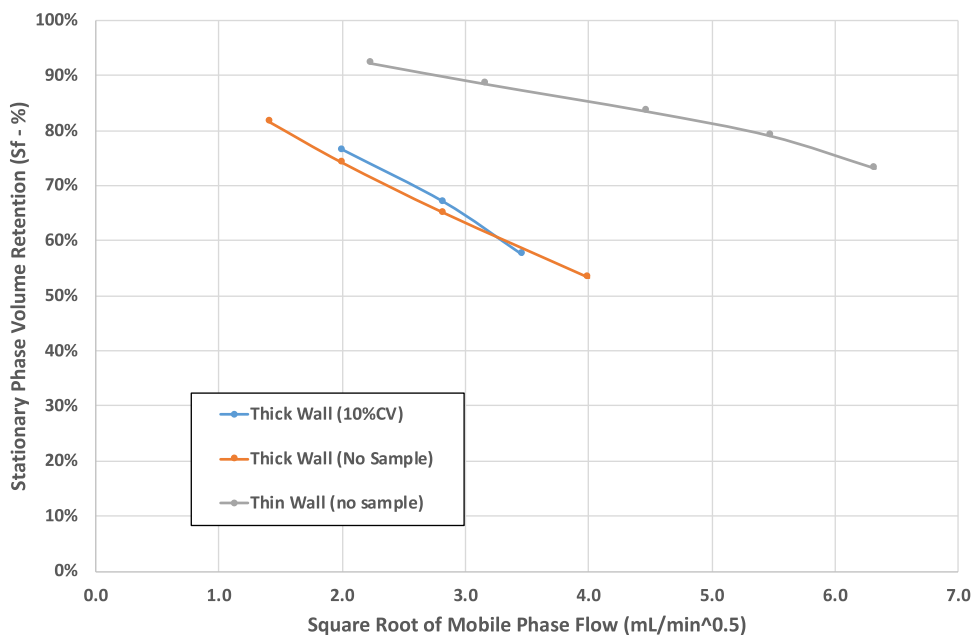
Fig. 1. Stationary Phase Volume Retention (Sf) v. Sqrt (F) for Rectangular Cross-section Thick and Thin-Wall Columns with Aspect Ratios of 3.125 and Operating at 243 g.
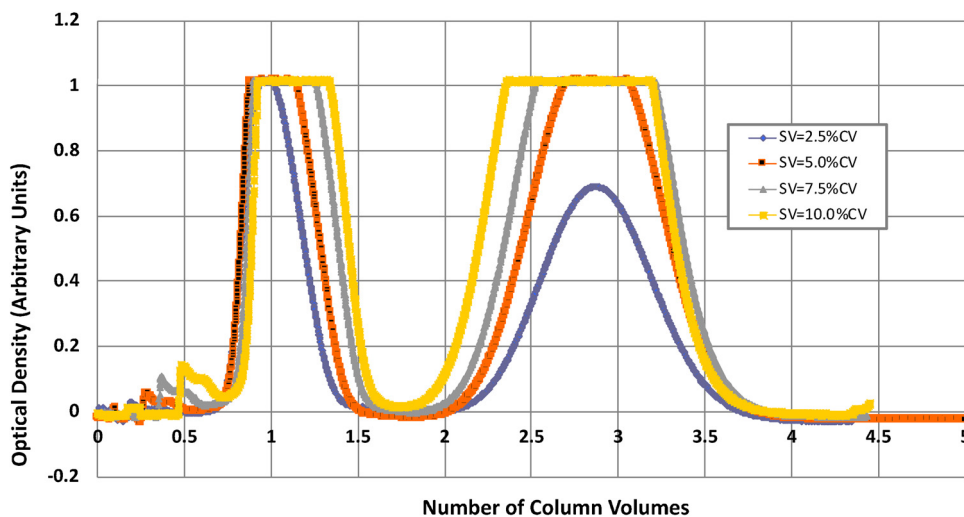

Fig. 2. Rectangular Thick Wall Horizontal Tubing Effect of Increasing Sample Volume. N = 2100 rpm, F = 2 ml/min, NP, HEMWat 5252, Vc = 24.3 mL.

optimization of mobile phase flow tests that follow.

3.3. The effect of mobile phase flow on baseline separation for the thick wall column

The effect of increasing mobile phase flow rate from 2 ml/min to 12 ml/min for rectangular thick wall horizontal tubing is shown in Fig. 3. Baseline separation is maintained for flows of 2, 4 and 8 ml/min, but at 12 ml/min baseline resolution is lost

3.4. The effect of mobile phase flow on baseline separation for the thin wall column

Baseline separation can be maintained for thick-wall tubing up to a mobile phase flow rate of 8 ml/min when the stationary phase volume retention (Sf) is 67% in Fig. 1. This suggests flow rates of 30 ml/min should be possible for thin-wall tubing as Sf = 69%, whereas at 40 ml/min Sf drops dramatically to 33%. This is shown to be the case in practice when mobile phase flow rate is increased
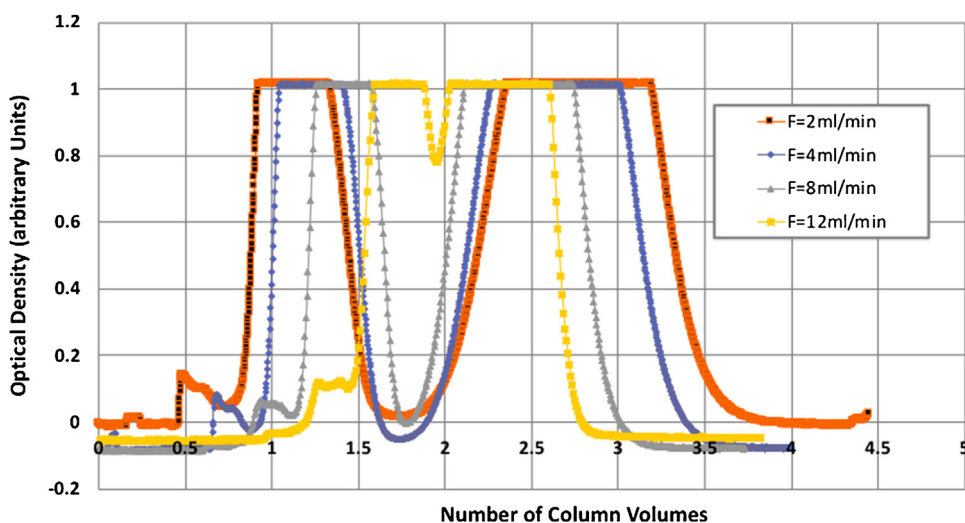
Fig. 3. Rectangular Horizontal Thick Wall Tubing - Effect of Flow for Sample Loading of 10%CV N = 2100 rpm, NP, HEMWat 5252, Vc = 24.3 mL.
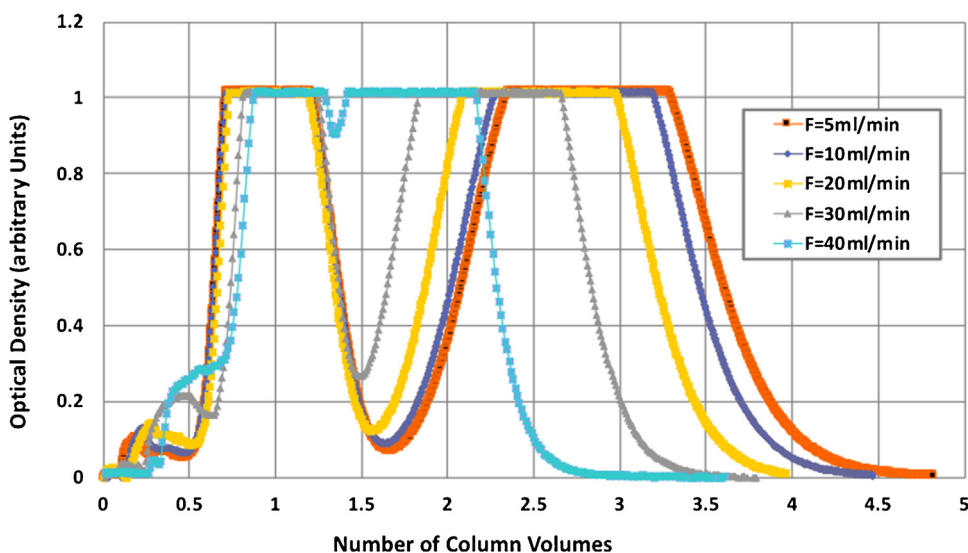

Fig. 4. Rectangular Horizontal Thin-Wall Tubing - Effect of Flow for Sample Loading of 10%CV N = 2100 rpm, NP, HEMWat 5252, Vc = 56 mL.

from 5 to 40 ml/min for the thin wall rectangular tubing column in Fig. 4. Near baseline separations are maintained up until a mobile phase flow of 30 ml/min but lost at 40 ml/min.

3.5. The variation of resolution between Honokiol and Magnolol with mean linear mobile phase flow

The variation of resolution between Honokiol and Magnolol is plotted against mean linear flow in Fig. 5. Resolution can be held above one to a much higher linear flow for the larger cross-sectional area thin-walled column than with the thick-walled column.

3.6. The variation of sample loading/throughput with mean linear mobile phase flow

The variation in sample loading/throughput in g/hour is shown in Fig. 6 plotted against mean linear mobile phase flow. Note that

these results are normalized to a resolution of Rs = 1.5. Throughput is proportional to mean linear flow for both the thick and thin-walled columns until at high linear flow a maximum is reached.

3.7. Overall improvement in performance over the standard commercial 18 ml, 0.8 mm bore column

The optimum throughput conditions for the thick and thin-wall tube columns are compared to the original standard column in Table 1. The thick and thin-wall columns give a 22x and 55x improvement over the original standard commercial column.

4. Discussion

The objective of this paper was to see how far the new more efficient column geometry combined with better packing efficiency with thinner wall tubing could increase sample loading and
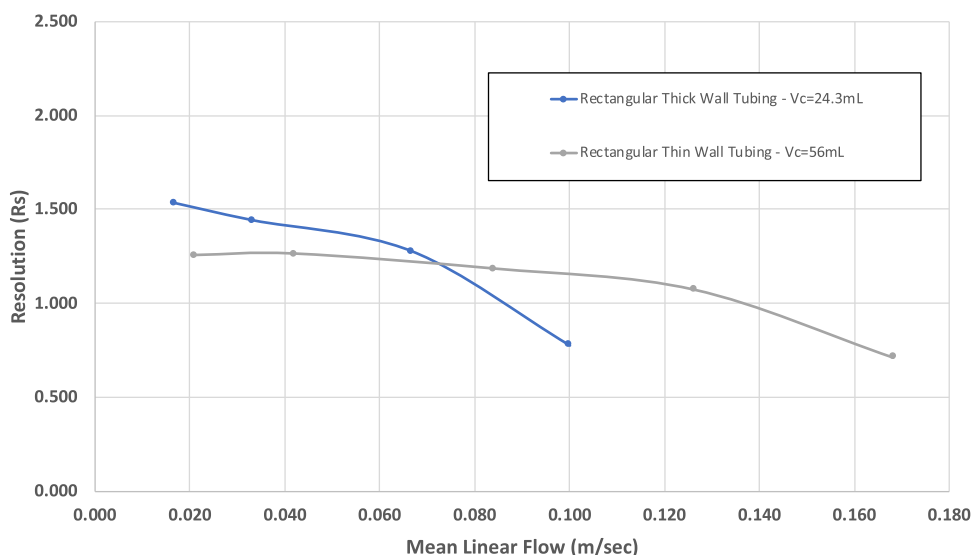
Fig. 5. Resolution (Rs) between Honokiol & Magnolol v. Linear Flow (m/sec) for Thick and Thin Wall Tubing, N = 2100 rpm, SV = 10%CV.
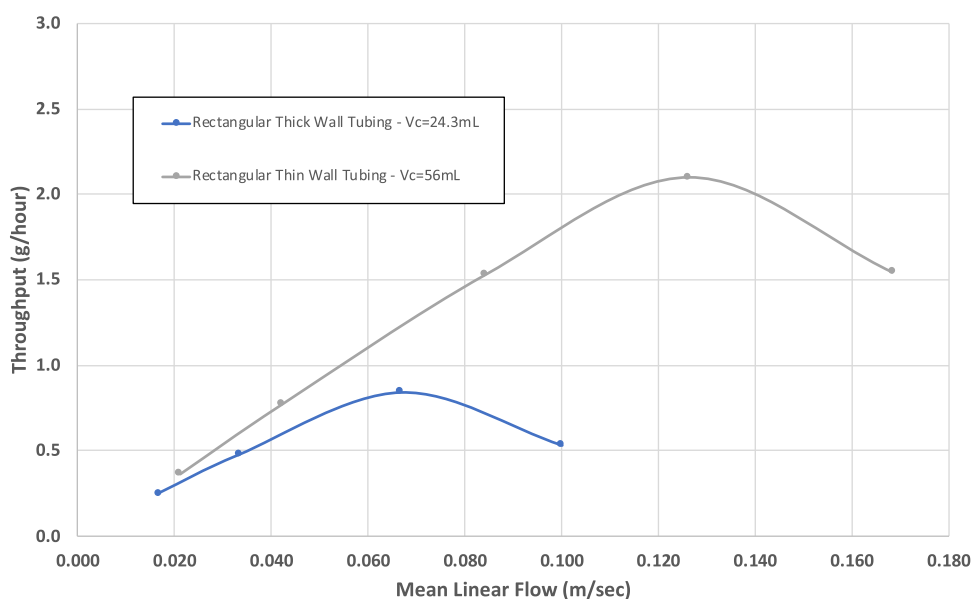

Fig. 6. Throughput (g/hour) v. Linear Flow (m/sec) N = 2100 rpm, SV = 10%CV.

Table 1
Improvement in throughput for thick and thin-wall tubing columns compared to the standard Mini analytical column.

Column	Bore or hxw (mm)	Aspect Ratio	Area (mm^2)	Volumn (mL)	Flow (mL/min)	Linear Flow (m/sec)	Sample Vol (CV%)	Resolution (Rs)	Throughput (g/h)	Throughput (g/h @ Rs = 1.5)	Improvement
Standard Circular Tubing Column	0.8	1.00	0.50	18.0	2.5	0.083	5	0.701	0.13	0.038	1.00
Thick Wall Tubing Column	0.8 × 2.5	3.13	2.00	24.3	8.0	0.067	10	1.277	1.16	0.844	22.21
Thin-wall tubing column	1.2 × 3.3	2.75	3.96	56.0	30.0	0.126	10	1.072	4.11	2.102	55.32

throughput from what was possible with the original commercial standard 18 ml column. Berthod [4] had already shown that increasing the bore of circular tubing could greatly increase stationary phase volume retention and allow faster separations with similar resolution despite the number of theoretical plates reducing dramatically. It should be emphasised that the same column bobbin/spool geometry was used so that the same analytical DE-Mini Instrument could still be used but with columns that would enable semi-preparative operation with the same resolution power. This was done by firstly by changing the geometry of the tubing from Berthod's 1.6 mm circular tubing column to a similar wall thickness (0.8 mm) rectangular column with an aspect ratio of 3.125 and internal dimensions 0.8 mm x 2.5 mm. Peng [3] had already shown that this arrangement could double column efficiency. It was found

that flows could be increased to 8 ml/min with a throughput of 0.84 g/h with a separation time of 10 min, which was a 22x improvement over the original sample loading optimisation [2] of 0.038 g/h at 2.5 mL/min with a separation time of 45 min.

Reducing the wall thickness of the tubing to 0.4 mm and maintaining the same outer dimensions gives a column of the same length but increases the volume from 24.3 mL to 56 mL and effectively doubles the cross-sectional area from 2 mm^2 to 3.96 mm^2 equivalent to a circular tubing column bore of 3.2 mm. It was found that the optimum flow of 30 mL/min could be predicted from the retention curves in Fig. 1. The throughput was 2.1 g/h with a separation time of only 6.5 min giving an overall improvement of 55x the original sample loading optimisation [2] of 0.038 g/h with a separation time that is 7x quicker.

What is amazing is that good resolution between Honokiol and Magnolol can be preserved as column volume increases and separation times are reduced. The original resolution [2] was 0.7 with the 0.8 mm bore circular tubing column and a flow of 2.5 mL/min (mean linear flow 0.083 m/sec). This increased to 1.28 for the thick-wall rectangular column at 8 mL/min (mean linear flow 0.067 m/sec) and only reduced to 1.07 for the thin-wall rectangular column at 30 mL/min (mean linear flow 0.126 m/sec).

This research opens the possibility of small analytical instruments being developed with high aspect ratio rectilinear columns, already shown to double column efficiency [3] compared to conventional circular columns, with a range of columns. These would vary in cross-sectional area depending on whether long column, small cross-sectional area analytical results are required where sample volumes are limited or shorter, large cross-sectional area semi-preparative columns where gram quantities of material can be harvested for further analysis.

It should be noted that, at this stage, the optimization has been demonstrated on analytical columns. The next step will be to examine whether similar improvements can be made at the preparative and industrial scale where further sample optimization strategies, as outlined by Kostanian [8], can be applied.

5. Conclusions

This paper demonstrates that changes in column geometry from conventional circular tubing to rectangular horizontal tubing of high aspect ratio where the wider side is perpendicular to the 'g' field, not only doubles column efficiency [3] but also increases sample loading capacity or throughput by up to 55 times the optimum conditions obtained using the conventional 0.8 mm 18 mL commercial column. Furthermore, this was achieved without changing the outside dimensions of the column and as large volumes of PTFE have been replaced by solvent system, the overall weight of the rotating bobbin has become lighter opening up the prospect of higher 'g' more efficient instruments being produced by manufacturers.

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